E.6

ENZYME INHIBITION IN DRUG DISCOVERY AND DEVELOPMENT

The Good and the Bad

Edited by

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EVALUATION OF INHIBITORS OF DRUG METABOLISM IN HUMAN HEPATOCYTES

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13.1 INTRODUCTION

One major challenge in the selection of drug candidates for clinical trials is that, due to species–species differences in drug properties, human-specific drug effects cannot be detected using nonhuman animal experimental systems. The high rate of clinical trial failures has been attributed to this species–species difference (DiMasi et al., 2003). One of the reasons for species–species differences in drug properties is the occurrence of species–specific xenobiotic metabolism pathways. Species differences in P450-dependent monooxygenases, a major group of enzymes responsible for drug metabolism, are well established (Guengerich, 2006) (Table 13.1).

In vitro experimental systems with human-specific properties represent an attractive tool for the assessment of human-specific drug properties. *In vitro* experimental systems derived from the human liver, namely, human hepatocytes and human liver tissue fractions, are now used routinely for the assessment of human drug metabolism. The combined use of human *in vitro* hepatic systems and relevant nonhuman animal models is believed to be responsible for the reduction in the contribution of pharmacokinetics as a major factor in human clinical trial failures from approximately 40% in 1991 to approximately 10% in 2000 (Kola and Landis, 2004).

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TABLE 13.1. Predominant P450 Isoforms in Various Animal Species[a]

	P450 Isoforms				
P450 Family	Human	Mouse	Rat	Dog	Monkey
CYP1A	1A1/2	1A1/2	1A1/2	1A1/2	1A1/2
CYP2A	2A6	2A5	2A5	2A13/25	2A23/24
CYP2B	2B6	2B9/10	2B1	2B11	2B17
CYP2C	2C8/9/19	2C29/37/38/40/44	2C6/7/11	2C21/41	2C20/43
CYP2D	2D6	2D22	2D1	2D15	2D17
CYP2E	2E1	2E1	2E1	2E1	2E1
CYP3A	3A4/5	3A11/13	3A1/2	3A12/26	3A8

[a]The P450-dependent monooxygenases are the major xenobiotic metabolizing enzymes in the liver. The table here illustrates one of the scientific bases for species-species differences in drug properties. Species specific P450 isoforms may lead to different affinities and rates of metabolism of xenobiotics, leading to species differences in metabolic fate and/or toxicity. For the five animal species shown here, species differences in P450 isoforms are present for all P450 families except for CYP1A and CYP2E.

For metabolism and drug–drug interaction studies, the human-based *in vitro* systems include cell-free systems such as liver homogenates, post-mitochondrial supernatants (S-9 or S-10), and purified liver microsomes. Liver microsomes, due to their ease of use and relatively low cost, are an experimental system of choice for the evaluation of metabolic stability, metabolite profiling, metabolite identification, and P450 inhibition studies. Genetically engineered microsomes (cDNA-expressed microsomes) with only one specific P450 isoform are used for the evaluation of isoform-specific properties (Li, 2001, 2004a).

There are limitations to the use of cell-free systems. Specifically, the use of liver microsomes excludes experimentation with enzymes present in the plasma membranes, mitochondria, and cytosol, which may play important roles in the metabolism of the drugs being studied. Furthermore, the lack of an intact plasma membrane and the associated uptake transporters precludes the evaluation of selective distribution of drugs between the intracellular and extracellular compartments.

Intact hepatocytes may represent a physiologically more relevant experimental system than cell-free systems. The parenchymal cells of the liver, commonly known as hepatocytes, contain the majority of, if not all, hepatic xenobiotic biotransformation enzymes. Hepatocytes isolation techniques were developed in the 1970s, and the isolated hepatocytes were proposed to be used as a relevant experimental system for the evaluation of drug properties (e.g., Fry, 1982), a view that continues to be held by the scientific community (e.g., Li, 2007; Gomez-Lechon et al., 2007). The use of hepatocytes in the evaluation of drug metabolism, drug–drug interaction potential, and drug toxicity is now routine practice in both academic and industrial laboratories (Li, 2005; Lu et al., 2007).

In this chapter, the use of hepatocytes in the evaluation of enzyme inhibition will be reviewed. Our discussion will be limited to the application of human hepatocytes, although the principles described here are also applicable to hepatocytes from laboratory animals.

13.1.1 Human Hepatocytes for the Evaluation of Human Drug Properties

Hepatocytes isolated from human livers represent a valuable experimental system in drug development. For decades, efforts in drug development have been handicapped by the inability in the accurate prediction of human *in vivo* drug properties in preclinical studies with laboratory animals. This human–nonhuman animal differences in drug-properties are mainly a result of species differences, especially in drug-metabolizing enzyme activities. As illustrated in Table 13.1, the isoforms of the major family of drug-metabolizing enzymes, the P450-dependent monooxygenases, are different between laboratory animals and humans. The different isoforms may lead to differences in rates of metabolism and formation of different metabolites from a chemical entity, resulting in species–species differences in metabolic fate and toxicity. A clear example of species differences in metabolism is the formation of 7-hydroxycoumarin in humans but not in rodents (Easterbrook et al., 2001).

Hepatocytes isolated from human livers would retain human-specific hepatic metabolism activities and therefore represent a valuable preclinical experimental system for the early assessment of human-specific drug properties.

13.1.2 Isolation of Human Hepatocytes

The general procedures for the isolation of hepatocytes from all animal species, including humans, are essentially similar, involving firstly the perfusion of the liver or liver fragment with an isotonic, divalent ion-free buffer containing the calcium chelator EGTA to remove blood, dissolve clots, and loosen cell–cell junctions. This is followed by perfusion with a collagenase-containing isotonic buffer with the divalent ions calcium and magnesium, which are required for collagenase activity. The collagenase serves as an enzyme to dissociate the hepatocytes from the liver parenchyma into single cell suspension. Our laboratory represents one of the first to isolate and cryopreserve human hepatocytes, and a detailed procedure for human hepatocyte isolation based on the original method of Berry and Friend (1969) was previously reported by our laboratory (Li et al., 1992) for human liver fragments. The procedures have now been modified for large-scale hepatocyte isolation from the whole human liver (Li, 2007).

Procurement of human livers for research as well as human hepatocyte isolation are activities that are not commonly available to most laboratories, and they had represented the major hindrance to research with human hepatocytes when this experimental system was initially introduced to the scientific

community. This major hindrance to the use of human hepatocytes is now circumvented by the cryopreservation of the hepatocytes.

13.1.3 Cryopreservation of Human Hepatocytes

Our laboratory was one of the first to report successful cryopreservation of human hepatocytes (Loretz et al., 1989), as well as the first to show (a) similar drug-metabolizing enzymes between cryopreserved and freshly isolated human hepatocytes and (b) the development of assays for metabolic stability, drug–drug interactions, and cytotoxicity using cryopreserved human hepatocytes (Li et al., 1999a,b). The similarity between freshly isolated and cryopreserved human hepatocytes in drug-metabolizing enzyme activities is now generally accepted by the scientific community (Li et al., 1999a,b; Li, 2007; Hewitt et al., 2007; Jouin et al., 2006).

Until recently, cryopreserved hepatocytes in general would lose their ability to be cultured as attached, monolayer cultures, presumably due to the unavoidable membrane damage occurring during the cryopreservation and subsequent thawing processes. It has been projected in the past that one out of 10–20 human hepatocyte isolations would lead to "plateable" cryopreserved hepatocytes. A focused research effort was initiated in our laboratory in 2005 to overcome this deficiency in hepatocyte cryopreservation. Our research resulted in the development of highly optimized hepatocyte isolation, cryopreservation, and recovery procedures (Li, 2007). In our laboratory, approximately 50% of the isolations would lead to "plateable" cryopreserved hepatocytes (Li, 2007).

Besides the retention of high viability and plateability, human hepatocytes after cryopreservation have been shown to retain human drug-metabolizing enzyme activities, including the activities of P450 isoforms, UDP-dependent glucuronosyl transferase activity (UGT), and sulfotransferase activity (ST) (Li et al., 1999a,b). The originally proposed applications of cryopreserved hepatocytes in drug metabolism studies (Li et al., 1999a,b) have been generally accepted by the scientific community at large (Jouin et al., 2006; Brown et al., 2007). Plateable cryopreserved human hepatocytes can also be used for enzyme induction studies (Kafert-Kasting et al., 2006; Hewitt et al., 2007). Cryopreserved human hepatocytes are found to retain uptake transporters such as Na^+-taurocholate cotransporting polypeptide (NTCP), organic anion transporting polypeptide (OATP), and organic cation transporter (OCT) (Shitara et al., 2003; Maeda et al., 2006). Besides the retention of uptake transporter activities, cryopreserved human hepatocytes were found, upon multiple days of culture, to form functional bile canaliculi and have been applied toward the evaluation of efflux transporter activities (Bi et al., 2006; Li et al., 2008).

Human hepatocyte cryopreservation is an enabling technology for the use of human hepatocytes. The advantages of cryopreserved hepatocytes over freshly isolated cells include long-term storage, ease of experimental

scheduling, choice of precharacterized lots for experimentation, and repeat experimentations with hepatocytes from the same donors. Thus, the U.S. FDA has listed in the latest guidance document (FDA, 2006) that cryopreserved human hepatocytes are an acceptable experimental system for the generation of drug metabolism and drug–drug interaction data to support IND and NDA submissions.

13.1.4 Prepooled Cryopreserved Human Hepatocytes from Multiple Donors

Cryopreserved human hepatocytes were first available commercially in the mid-1990s. Since then, this experimental system is widely accepted by the pharmaceutical industry in drug development studies. Until the early 2000s, cryopreserved hepatocytes from individual donors were used. Recognizing that for the evaluation of "general" drug properties such as metabolic stability and metabolite profiling, one would like to have results representing the average of multiple individuals, practitioners of the field would pool cryopreserved hepatocytes from multiple donors for their studies. This is akin to the use of liver microsomes that are prepared from multiple individuals.

To eliminate the need to thaw hepatocytes from multiple donors, it is now discovered that cryopreserved human hepatocytes can be thawed, pooled, and recryopreserved without significant changes in viability or drug-metabolizing enzyme activities. These prepooled human hepatocytes (usually pooled from five male and five female donors) are now available commercially for experimentation. The viability, cell morphology, and enzyme activities of one of the lots are illustrated in Fig. 13.1. The ability to prepare prepooled human hepatocytes represent another major advance in hepatocyte technology, allowing this experimental system to be used to replace liver microsomes in studies that the use of hepatocytes represent a more relevant approach.

13.1.5 Applications of Human Hepatocytes in Drug Development

The following are the current routine applications of human hepatocytes in drug development. Cryopreserved human hepatocytes, especially prepooled human hepatocytes, are recommended to be used for these assays. The general scientific principles of the *in vitro* screening methodologies have been previously reviewed (Li, 2001, 2004a, 2007), the specific procedures for the assays are described here.

1. *Metabolic Stability Screening.* An important "drug-like" property for new chemical entities (NCE) is appropriate metabolic stability to allow a practical frequency of drug administration. In the past, liver microsomes were used routinely for metabolic stability screening. However, as liver microsomes contain mainly enzymes such as the P450 isoforms for Phase I oxidation, the assay would only yield metabolic stability toward microsomal oxidative enzyme metabolism, while in humans *in*

HuP58 -Human -10 Donors Pool (5 Male; 5 Female)

INVENTORY		CELL BIOLOGY (CHRM Method)			METABOLISM PROFILE pmol/1 x 10^6 cells/min							
LOT#	VIALS	Initial viability (%)	YIELD (Viable cells per vial)	Viability after 2 hrs at 37 deg. C (%)	ECOD	7-HCG/S	CYP 1A2	CYP 2C8	CYP 2C9	CYP 2C19	CYP 2D6	CYP 3A4
HuP58	469	81%	5.8 Million	71%	21	33	38	34	83	82	16	567

DONOR INFORMATION		
GENDER	RACE	AGE
M	C	39
M	C	41
M	C	56
M	C	59
M	C	20
F	C	18
F	C	17
F	C	26
F	C	53
F	C	17

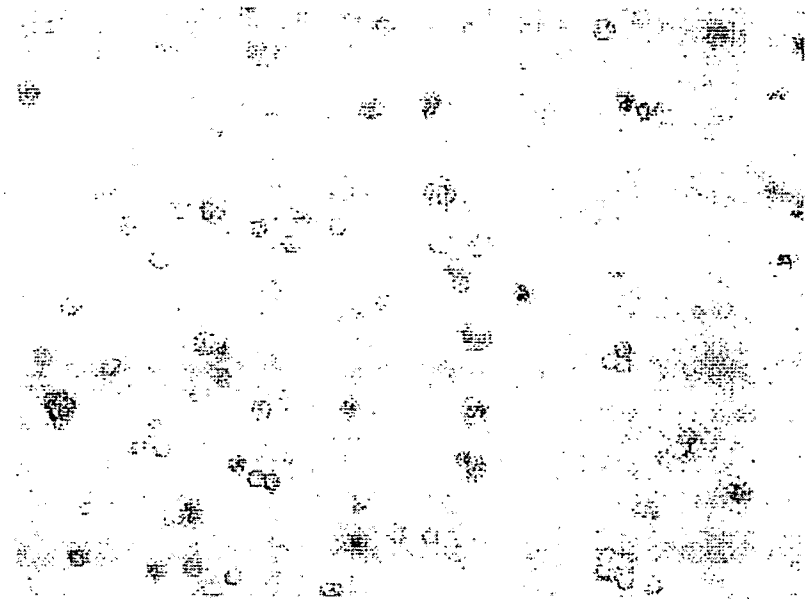

Figure 13.1. Viability, drug metabolic enzyme activities, and cell morphology of thawed prepooled cryopreserved human hepatocytes. For the preparation of prepooled cryopreserved human hepatocytes, cryopreserved cells from 10 donors (five male, five female) were thawed, pooled, and recryopreserved. The figure here shows the properties of the cells thawed after the second cryopreservation. The results show that although the cells have been cryopreserved and thawed twice, they retain their typical morphology, high viability, and normal drug-metabolizing enzyme activities. The age and race of the donors are also shown. Viability was determined by trypan blue exclusion. ECOD, 7-ethoxycoumarin-*O*-deethylase activity; 7-HCG/S, 7-hydroxycoumarin glucuronidation and sulfation activities. Figure courtesy of Invitrogen CellzDirect.

vivo, the chemicals studied may be cleared via nonmicrosomal enzyme pathways such as conjugating enzyme pathways. Intact hepatocytes therefore represent a more relevant experimental system for metabolic stability evaluation than liver microsomes (Lavé et al., 1997; Li, 2001, 2004a, 2007; Jouin et al., 2006).

2. *Metabolite Profiling and Species Comparison.* The identification of metabolites formed from the parent drug (metabolite profiling) is important to drug development, because it allows the design of chemical structure to improve metabolic stability or to decrease cytotoxicity (see below). Metabolite identification is also important for the determination of the key drug metabolizing-enzyme pathways (e.g., oxidation or conjugation) as part of the program to understand drug–drug interaction potential. Lastly, metabolite profiling allows the selection of laboratory animal species most relevant to humans for *in vivo* experimentation. An animal species that forms metabolites found in humans would be more relevant than one with metabolites different from those formed in humans. This species comparison is routinely performed using *in vitro* systems such as hepatocytes (e.g., from human, rat, mouse, guinea pig, dog, monkey) (Lee et al., 1994; Li, 2001, 2004a,b; Zhang et al., 2007).
3. *Drug–Drug Interaction Evaluation.* A major adverse drug property with fatal outcome is drug–drug interaction. A drug may inhibit the metabolic clearance of a co-administered drug, leading to toxicity due to high systemic exposure to the affected drug (inhibitory drug–drug interactions). Conversely, a drug may enhance the metabolic clearance of a coadministered drug, leading to inefficacy due to lower than optimal systemic exposure (inductive drug–drug interactions). Inhibitory drug–drug interactions are caused by the inhibition of drug-metabolizing enzyme activities. Inductive drug–drug interactions are caused by the induction of drug-metabolizing enzyme activities. Both types of drug–drug interactions can be evaluated with human hepatocytes (Li, 2001, 2004a,b)

13.1.6 Evaluation of the Inhibition of Drug-Metabolizing Enzymes in Human Hepatocytes

In this review, emphasis is placed on the use of human hepatocytes for enzyme inhibition studies. The use of human hepatocytes for enzyme induction studies have been reviewed recently (Hewitt et al., 2007). As discussed earlier, intact hepatocytes represent an ideal experimental system for the evaluation of drug metabolism. For the same reasons, they also represent an ideal experimental system for the evaluation of the inhibition and induction of drug-metabolizing enzymes, either in the context of drug–drug interactions or in toxicity evaluation. The advantages of the use of intact hepatocytes to evaluate the inhibitory potential of a chemical on drug-metabolizing enzymes are as follows (Li, 2007):

1. *Intact Plasma Membranes.* Chemicals that are not permeable to the plasma membranes will not be available to intracellular enzymes. Intact hepatocytes have intact plasma membranes and therefore will model differential distribution between extracellular and intracellular environments as occur *in vivo*. Cell-free systems such as liver microsomes do not have a biological barrier between the chemicals to be evaluated and the drug-metabolizing enzymes and may lead to nonphysiological interactions between the nonpermeable chemicals and intracellular enzymes.
2. *Active Uptake Transporters.* Besides the intact plasma membranes, intact hepatocytes have active uptake transporters that can actively accumulate a chemical, leading to a higher intracellular than extracellular concentrations. This differential distribution may lead to a higher inhibitory potential than would be expected from the plasma (extracellular) concentrations. Furthermore, drug–drug interactions may also occur at the site of the active transporters that can be studied with intact hepatocytes.
3. *Complete Drug-Metabolizing Enzyme Pathways.* The effects of an enzyme inhibitor are determined by its concentration at the active site of the affected enzyme. Two of the determinants of intracellular enzyme-site concentrations, as discussed earlier, are its permeability and, if it is a substrate, uptake transporter activity. A third determinant is its metabolism by intracellular enzymes. For instance, an inhibitor may be rapidly conjugated by glucuronosyl transferases or sulfotransferases to metabolites that have lower or no inhibitory activities. In some cases, these metabolites may demonstrate higher inhibition potential. Metabolism by these or other cytosolic enzymes cannot be effectively modeled with liver microsomes.
4. *Physiological Enzyme and Cofactor Concentrations.* One major advantage of intact hepatocytes is that enzymes and cofactors are present in physiological concentrations. Furthermore, the multiple enzyme systems are present uninterrupted unlike other cell-free systems that are prepared by homogenization of the liver.

13.2 P450 INHIBITORS ON DRUG METABOLIZING ENZYME ACTIVITIES IN HUMAN HEPATOCYTES

Evaluation of P450 inhibition can be performed readily using cryopreserved human hepatocytes prepooled from multiple donors. The P450 isoform-specific substrates used routinely for the inhibitory drug–drug interaction assay and isoform-specific inhibitors that can be used as positive controls for the assay are shown in Table 13.2. The procedures for hepatocyte P450 inhibition assay is as shown below:

1. Add 125 μL of HMM containing 4× concentration of the drug to be evaluated into each well of a 24-well plate.

TABLE 13.2. Isoform-Selective Substrates and Their Respective Metabolites and Inhibitors that Can Be Used as Positive Controls for the Cryopreserved Human Hepatocyte P450 Inhibition Study[a]

P450 Isoform	Substrate	Metabolite	Positive Control
1A2	Phenacetin	Acetaminophen	Furafylline
CYP2A6	Coumarin	7-OH Coumarin	8-Methoxypsoralen
CYP2B6	Buproprion	Hydroxybuproprion	Triethylenethiophosphoramide (ThioTEPA)
CYP2C8	Paclitaxel	6a-OH Paclitxel	Quercetin
CYP2C9	Tolbutamide	4-OH Tolbutamide	Sulfaphenazole
CYP2C19	*S*-Mephenytoin	4-OH Mephenytoin	Ticlopidine hydrochloride
CYP2D6	Dextromethorphan	Dextrophan	Quinidine
CYP2E1	Chlorzoxazone	6a-OH Chlozoxazone	Diethyldithiocarbamate
CYP3A4	Testosterone	6b-Hydroxytestosterone	Ketoconazole

[a]It is recommended that prepooled cryopreserved human hepatocytes are used for this assay, akin to the use of liver microsomes pooled from multiple donors. The intact hepatocytes provide intact cell properties including an intact plasma membrane with uptake transporter activities as well as complete, uninterrupted enzyme pathways at physiological cofactors that are not present in liver microsomes.

2. Add 125 μL of HMM containing 4× concentration of the drug-metabolizing enzyme substrate into the same well.
3. Add 250 μL of HMM containing 250,000 human hepatocytes.
4. Incubate for 30 min at 37 °C.
5. Add 1 mL of ACN to terminate reaction.
6. Centrifuge to remove cellular macromolecules.
7. LC/MS or HPLC quantification of metabolites.

13.2.1 Towards a More Physiological Model: Human Hepatocyte/Whole Plasma System for P450 Inhibition

A major challenge of *in vitro* drug–drug interaction studies is to predict the extent of clinical drug–drug interactions. A common approach is to use the ratio of the inhibitor's physiological concentration [I] over the inhibitor's inhibition constant $K_i([I]/K_i)$ (Soars et al., 2003; Ito et al., 2004; Blanchard et al., 2004; Cook et al., 2004; Obach et al., 2006; Bachmann, 2006; Galetin et al., 2006). This simplistic approach suffers the drawback of the difficulties of obtaining accurate [I] and K_i values.

Theoretically, K_i is an absolute value dependent only on the inhibitor–enzyme affinity. However, there is substantial difficulty in the accurate determination of K_i values. The determined K_i value, or so-called apparent K_i value, is known to be influenced by the following factors: (1) *Ubiquitous protein binding*: For inhibitors that have high protein-binding potential, the amount of inhibitor available for interaction with the enzyme active site can be reduced

due to nonspecific binding (ubiquitous binding). What has been observed is that for inhibitors that are highly protein-bound, the higher the liver microsomal concentration is used, the higher would be the apparent K_i values. (2) *Enzyme substrates*: Different substrates often generate different K_i values. (3) *Enzyme system*: Different systems, for instance, recombinant enzymes and human liver microsomes, could generate different K_i values. Thus, the K_i values for ketoconazole in the literature vary from 0.015 to 8μM (Thummel and Wilkinson, 1998), a range of 500-fold. The K_i values for ketoconazole listed in the recently published FDA Drug–Drug Interaction Draft Guidance (2006) also carry a 50-fold difference range from 0.0037 to 0.18μM.

In addition to the difficulty in determining a reliable value of K_i, the determination of physiological inhibitor concentration [I] is also extremely difficult. While plasma [I] can be experimentally measured, what is needed is not plasma [I], but [I] at the active site of the enzymes to be studied. As discussed earlier, intracellular hepatic concentration of an inhibitor is affected by its binding to plasma proteins, active transporter activities (if it is a uptake transporter substrate), and intracellular metabolism by hepatic enzymes. While various methods have been employed to estimate [I] (Ito et al., 2004; Blanchard et al., 2004; Cook et al., 2004; Obach et al., 2006; Bachmann, 2006), not a single approach could be applied to different classes of drugs.

Because of the difficulties of accurately measuring both [I] and K_i, the use of the ratio of these two parameters, [I] / K_i, to predict drug–drug interaction is often found to yield results that are not quantitatively representative of the *in vivo* situation. To overcome this challenge, a novel experimental model, the hepatocytes suspension/whole plasma system, has been developed (Lu et al., 2006). Because this model encompasses the key *in vivo* parameters—(a) plasma proteins to account for protein binding, (b) intact hepatocytes to allow partitioning across the intact plasma membrane, and (c) uptake transporter and complete drug-metabolizing enzyme pathways—the observed results should represent *in vivo* inhibitory potential. The human hepatocyte–plasma system accurately predicted drug–drug interaction effects of ketoconazole (Lu et al., 2008a) and fluconazole (Lu et al., 2008b) and is believed to be applicable to most inhibitors. One drawback of the assay is that hepatocytes in suspension are known to lack efflux transporters. Efflux pump substrates that can be pumped out of hepatocytes, leading to reduced hepatocyte concentration and hence reduced potency, therefore cannot be effectively studied.

The predicted drug–drug interaction for ketoconazole and fluconazole as compared to actual clinical findings are shown in Tables 13.3 and 13.4, which were modified from Lu et al. (2008a,b).

13.2.2 Inhibition of Drug-Metabolizing Enzymes in Hepatotoxicity Screening

Hepatotoxicity is a major manifestation of drug toxicity, because the liver usually would receive the highest bolus concentration of an ingested drug.

TABLE 13.3. Drug–Ketoconazole DDI Prediction Using the Hepatocytes in Plasma Model[a]

Compound	Fold of AUC Change		f_{renal}
	Predicted	Observed	
Theophylline	1.13–1.16	1.11	0.18
Desipramine	1.00	1.02	0.70
Midazolam	16.7	17.0	<0.01
Tolbutamide	1.43	1.77	0.001
Omeprazole	1.74–2.74	1.36–2.05	<0.01
Loratadine	2.48	3.47	Negligible
Cyclosporine	3.45	4.39	<0.01
Alprazolam	2.75–4.91	3.98	0.20
Budesonide	5.11	5.39	Negligible
Buprenorphine	5.17	2.30	Negligible
Docetaxel	3.48	2.22	0.02
Loratadine	1.92	3.47	Negligible
Methylprednisolone	2.19	2.36	0.05
Sirolimus	8.66	10.9	Negligible
Tacrolimus	1.95	2.39	<1

[a]The fold AUC change was calculated based on data obtained *in vitro* using human hepatocytes suspended in whole human plasma as described in Lu et al. (2008). The results illustrate the accuracy of this experimental system in the prediction of *in vivo* effects, which is attributed to the presence of whole plasma for the modeling of plasma factors and intact human hepatocytes for the modeling of intact cell properties.

TABLE 13.4. Drug–Fluconazole DDI Prediction Using the Hepatocytes in Plasma Model[a]

Compound	Fold of AUC Change		Prediction Error (%)	f_{renal}
	Predicted	Observed		
Theophylline	1.00	1.19	−16.0	0.18
Tolbutamide	2.44	2.09	16.5	0.001
Omeperazole	2.61	6.29	−58.5	<0.01
S-Wafarin	2.76	2.84–4.31	−22.9	<0.02
Phenytoin	2.19	1.75	25.1	0.02
Midazolam	2.89	3.60	−19.7	<0.01
Sirolimus	3.02	4.70	−35.7	Negligible
Cyclosporine	1.98	1.84	7.6	<0.01
Tacrolimus	1.57	1.19	31.8	<0.01

[a]The fold AUC change were calculated based on data obtained in vitro using human hepatocytes suspended in whole human plasma as described in Lu et al. (2008b). The results illustrate the accuracy of this experimental system in the prediction of *in vivo* effects, which is attributed to the presence of whole plasma for the modeling of plasma factors and intact human hepatocytes for the modeling of intact cell properties.

Furthermore, the hepatocytes, being the cells responsible for drug metabolism, are the first cells to be affected by reactive or toxic metabolites. Isolated hepatocytes therefore represent a physiologically relevant experimental model for the evaluation of hepatotoxicity. *In vitro* hepatocyte cytotoxicity measurements have been found to be effective in the delineation of hepatotoxic and help to find less hepatotoxic structures (Li, 2007).

While drug metabolism is generally believed to be an important parameter of drug toxicity, it is often difficult to ascertain the role of drug metabolism in the observed adverse drug effects. One approach is to identify the metabolites for experimental evaluation of the toxicity of the metabolites. This approach, however, has the following drawbacks:

1. *Expense.* Extensive resources are needed for the identification and subsequent purification or manufacturing of the metabolites for testing.
2. *Relevance.* Testing of the observed metabolites may or may not reflect the toxic mechanisms *in vivo*. Firstly, the final metabolites may or may not represent the toxic metabolite: The toxic species may be a highly reactive, relatively unstable metabolite or intermediate. Secondly, due to reactivity and membrane permeability, metabolites formed *in situ* may react with different cellular targets if added to extracellular media.

Building on the successes in using hepatocytes in drug metabolism, drug–drug interactions, and hepatotoxicity studies, two assays have been recently developed with plateable cryopreserved human hepatocytes for the evaluation of the role of metabolism in xenobiotic toxicity: The Metabolic Comparative Cytotoxicity Assay (MCCA) and the Cytotoxic Metabolic Pathway Identification Assay (CMPIA) (Li, 2009).

In the MCCA, the cytotoxicity of a drug is evaluated in the metabolically incompetent cell line [e.g., Chinese hamster ovary (CHO) cells] and in the metabolically competent primary human hepatocytes, in both the absence and presence of a potent cytochrome P450 (CYP) inhibitor, 1-aminobenzotriazole (ABT). ABT has been reported to cause autocatalytic inactivation of P450, leading to a nonspecific, mechanism-based inhibition of multiple human P450 isoforms including CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4. In the MCCA, a chemical that is transformed by metabolism to cytotoxic metabolites would be more cytotoxic in the metabolically competent cells than in the metabolically incompetent cells. Furthermore, if P450-dependent oxidative metabolism is involved in the metabolic activation, ABT would attenuate the cytotoxicity of this chemical in the metabolically competent cells.

The CMPIA is proposed to be performed when a toxicant is found to require xenobiotic metabolism in order to be cytotoxic in the MCCA. In this assay, the cytotoxicity of the toxicant is evaluated in human hepatocytes, in the presence and absence of isoform-selective P450 inhibitors, to define which P450 isoforms are involved in metabolic activation. If a specific metabolic

pathway is involved in metabolic activation, a selective inhibitor of this metabolic pathway is expected to attenuate the cytotoxicity of the chemical in question.

The MCCA was developed in our laboratory for the definition of the general role of hepatic metabolism, especially P450-dependent metabolism in cytotoxicity, and the CMPIA for the evaluation of the role of specific P450 isoforms in metabolic activation. Because our major interest is to define human drug toxicity, human hepatocytes—and, more specifically, plateable cryopreserved human hepatocytes—were used in these assays. Human hepatocytes are known to retain human-specific drug metabolism and therefore can be used for the evaluation for the evaluation of the role of human drug metabolism on drug toxicity. As a control, a metabolically incompetent cell, the CHO cell, is used for comparison with the metabolically competent human hepatocytes in these assays to further define metabolism-related cytotoxic effects.

The application of MCCA and CMPIA to evaluate the role of drug metabolism in the toxicity of a toxicant has been illustrated with aflatoxin B1 (AFB_1), a hepatotoxicant and hepatocarcinogen that is known to require P450-dependent drug metabolizing enzyme activities for its toxicity (Figs. 13.2A–13.2D; Li, 2009). In the MCCA, AFB_1 was found to be more cytotoxic in the metabolically competent human hepatocytes than in the metabolically incompetent CHO cells, therefore confirming the known requirement of hepatic metabolism for its hepatotoxicity (Figs. 13.2A and 13.2D). Furthermore, the nonspecific P450 inhibitor, ABT, was found to be effective in attenuating AFB_1 cytotoxicity, thereby confirming that P450-dependent mixed function monooxygenase activity is involved in metabolic activation (Fig. 13.2A). In the CMPIA, ketoconazole and diethyldithiocarbamate, but not furafylline, sulfaphenazole, nor quinidine, were found to be effective in attenuating AFB_1 cytotoxicity in human hepatocytes (Figs. 13.2B and 13.2C). Ketoconazole was found to be as effective as the nonspecific inhibitor, ABT, therefore suggesting that the pathways inhibited by ketoconazole (mainly CYP3A4) are key pathways for metabolic activation. As diethyldithiocarbamate is known to inhibit CYP2A6 and CYP2E1, the results suggest that one or both of these isoforms may also be involved in AFB1 activation. The lack of effects of furafylline, sulfaphenazole, and quinidine on AFB1 cytotoxicity suggest that CYP1A2, CYP2C9, and CYP2D6 are not key pathways for AFB1 activation. The negative results are important to aid in ruling out key isoforms for metabolic activation, because isoform-selective inhibitors, while selective for certain isoforms, are known to have effects on multiple pathways. The lack of effects by furafylline, sulfaphenazole, and quinidine combined with the attenuating effects of ketoconazole and diethyldithiocarbamate suggest that CYP3A4 (the major P450 isoform inhibited by ketoconazole) and CYP2E1 and/or CYP2A6 (the major P450 isoforms inhibited by diethyldithiocarbamate) are the major isoforms involved in the metabolic activation of AFB1. Our observation, that CYP3A4 is involved in the metabolic activation of AFB1 to

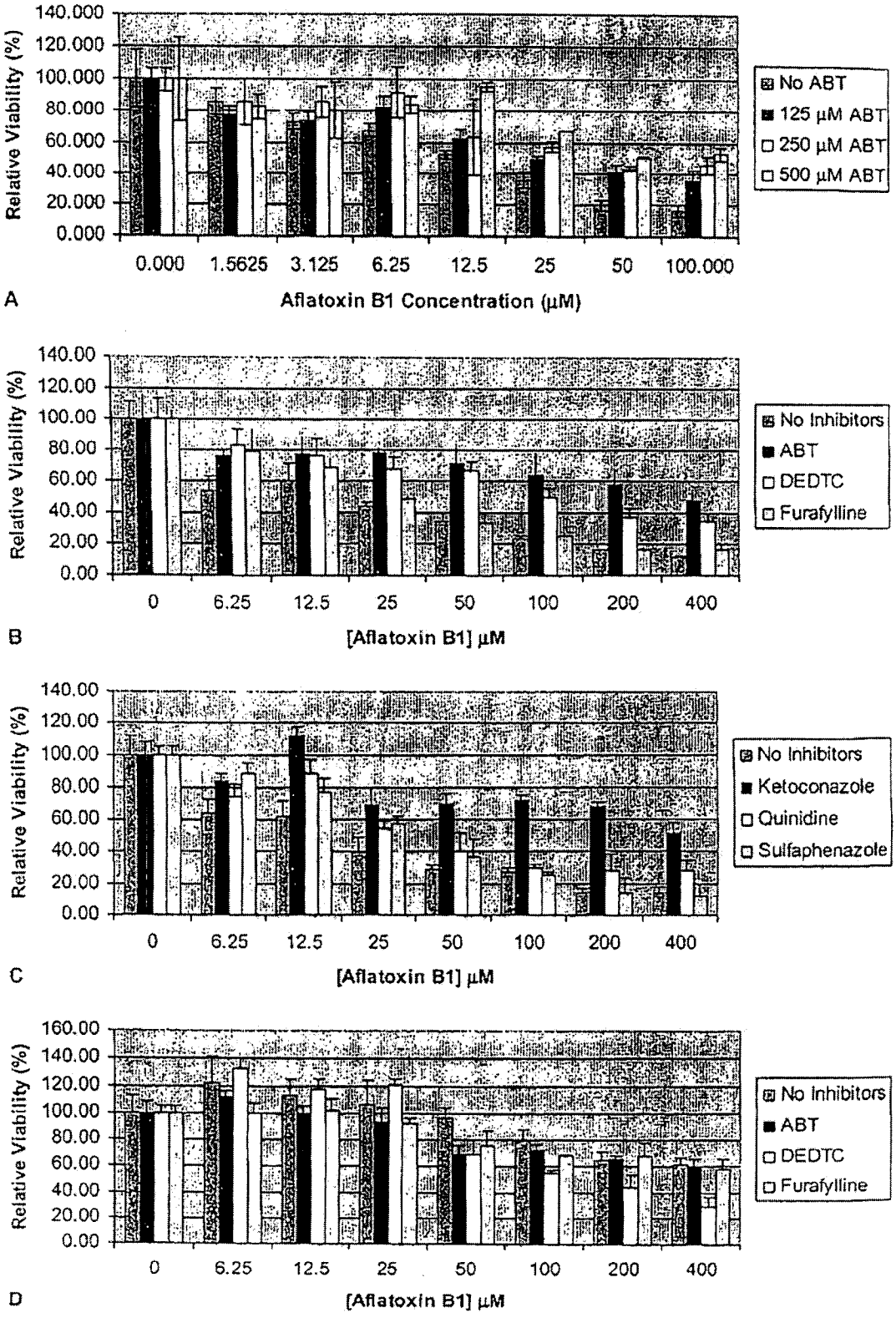

Figure 13.2. **(A)** Effect of ABT on aflatoxin B1 cytotoxicity in human hepatocytes. **(B)** Effect of ABT, EDDTC, and furafylline on aflatoxin B1 cytotoxicity in human hepatocytes. **(C)** Effect of ketoconazole, quinidine, and sulfaphenozole on aflatoxin B1 cytotoxicity in human hepatocytes. **(D)** Effect of ABT, EDDTC, and furafylline on aflatoxin B1 cytotoxicity in CHO cells.

cytotoxic metabolite, is consistent with the results obtained by others based on the quantification of toxic metabolites.

The results suggest that the MCCA and CMPIA are useful assays to estimate the role of xenobiotic metabolism, especially metabolism by P450 isoforms, in drug toxicity. Because P450-related pathways may not be the only pathways involved in metabolic activation, we are also developing approaches to evaluate non-P450 metabolic pathways such as alcohol dehydrogenase, esterase, monoamine oxidase, and flavin-dependent monooxygenases in drug toxicity in the MCCA and CMPIA. We believe that the MCCA and CMPIA can be used to identify drugs that would require metabolism to be toxic. These assays can be followed with analytical chemistry studies in metabolite identification for a definitive elucidation of the key pathways involved in the generation of toxic metabolites. Knowledge of the key pathways may allow the identification of human subpopulations that, due to genetic and environmental conditions, would be more susceptible to the toxicity of the drugs in question.

13.3 DISCUSSION

While this chapter deals with enzyme inhibition studies in human hepatocytes, the underlining theme is that one should always critically evaluate the tools used for drug development. To be able to predict accurately human *in vivo* effects, one needs to consider the key parameters and critically asks if they are adequately modeled in the chosen experimental system. The intact cell properties such as intact plasma membrane, active transporters, and complete, uninterrupted drug metabolizing enzyme pathways are properties in human hepatocytes that are not present in liver microsomes. Using intact human hepatocytes suspended in whole plasma is another step forward in terms of physiological relevance.

The big picture is that the earlier one can determine human-specific drug properties, the more likely is one to successfully develop a nontoxic and efficacious drug. An ideal drug candidate is one that is readily absorbed, has an acceptable plasma half-life to accommodate a convenient drug administration schedule, and has high efficacy, minimum toxicity, and minimum drug–drug interaction potential. Successful selection of drug candidates with these desired properties would greatly enhance the efficiency of drug development.

Because of species–species differences in drug properties, results with laboratory animals are not always predictive of human drug properties. The use of human-based *in vitro* experimental systems during preclinical trials allows the early assessment of human-specific drug properties. The reduction of pharmacokinetics as a contributing factor in clinical trial failures of drug candidates is attributed to the application of the *in vitro* hepatic system in the definition of human drug metabolism.

The successes of the hepatic *in vitro* systems underscore the importance of a science-based approach to the prediction of human drug properties—a key

step toward the enhancement of the efficiency of drug development. The scientific understanding of the basis of species–species differences in drug-metabolizing enzymes has argued strongly for the deficiency of nonhuman laboratory animal models and the relevance of human-based metabolically competent experimental systems. In past two to three decades, the persistence of the pioneers of the field in the painstaking characterization of the *in vitro* systems and the generation of supportive data has led to the endorsement of the U.S. FDA, which ultimately led to the universal acceptance of the use of *in vitro* approaches to define human drug metabolism and drug-drug interaction potential. The positive experience with *in vitro* hepatic systems has paved the way for an important goal of alternative experimental systems, namely, the reduction, refinement, and replacement of the use of nonhuman laboratory animals in the assessment of human drug toxicity. Human hepatocyte technologies—isolation, cryopreservation, prepooling, and applications in drug metabolism, enzyme inhibition, and enzyme induction—have proven to be critical scientific advancements that, when applied intelligently, will contribute to the ultimate success in the preclinical prediction of human drug properties.

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